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Induction of Adipocyte Complement-Related Protein of 30 Kilodaltons by PPARγ Agonists: A Potential Mechanism of Insulin Sensitization

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Adipocyte complement-related protein of 30 kDa (Acrp30, adiponectin, or AdipoQ) is a fat-derived secreted protein that circulates in plasma. Adipose tissue expression of Acrp30 is lower in insulin-resistant states and it is implicated in the regulation of *in vivo* insulin sensitivity. Here we have characterized the ability of PPARγ agonists to modulate Acrp30 expression. After chronic treatment of obese-diabetic (*db/db*) mice with PPARγ agonists (11 d), mean plasma Acrp30 protein levels increased (>3×). Similar effects were noted in a nongenetic type 2 diabetes model (fat-fed and low-dose streptozotocin-treated mice). In contrast, treatment of mice (*db/db* or fat-fed) with metformin or a PPARα agonist did not affect plasma Acrp30 protein levels. In a cohort of normal human subjects, 14-d treatment with rosiglitazone also produced a 130% increase in circulating Acrp30 levels *vs.* placebo. In addition, circulating Acrp30 levels were suppressed 5-fold in patients with severe insulin resistance in association with dominant-negative PPARγ mutations. Thus, induction of adipose tissue Acrp30 expression and consequent increases in circulating Acrp30 levels represents a novel potential mechanism for PPARγ-mediated enhancement of whole-body insulin sensitivity. Furthermore, Acrp30 is likely to be a biomarker of *in vivo* PPARγ activation. (*Endocrinology* 143: 998–1007, 2002)

MANY STUDIES HAVE shown that thiazolidinediones (TZDs) produce insulin sensitizing effects in animal models (1–4) and in insulin-resistant human subjects (5–7). It is now clear that TZDs are high affinity ligands for the nuclear receptor, PPARγ, which can transactivate PPAR-responsive gene promoters (8). Moreover, *in vivo* efficacy of TZDs in rodents generally correlates with *in vitro* PPARγ activity (2, 9). We and others have also discovered non-TZD PPARγ agonists with efficacy in rodent models of type 2 diabetes (10, 11). An important recent observation that serves to further validate PPARγ as a key controller of insulin sensitivity and glycemia was the finding of dominant-negative PPARγ gene mutations in two families with an inherited form of insulin-resistant type 2 diabetes (12).

Despite a wealth of knowledge pertaining to PPARγ functions, mechanism(s) that underlie the ability of PPARγ agonists to improve *in vivo* insulin sensitivity and hyperglycemia are not well understood. After chronic agonist therapy, skeletal muscle glucose disposal is improved (13, 14); in addition, insulin-mediated suppression of hepatic glucose output is enhanced (13) (Doebber, T., unpublished). However, it is doubtful that net improvements in whole body glucose homeostasis can be explained by direct actions of PPARγ in liver or muscle. In contrast to direct effects of PPARγ agonists to augment insulin action in cultured adipose tissue (15), we were unable to detect direct effects using isolated skeletal muscles from normal or insulin-resistant rodents (14). In addition, PPARγ expression levels in liver are very low (16), and there is no convincing evidence that PPARγ agonists have direct effects on hepatic insulin action. Therefore, we hypothesized that PPARγ agonists have predominant direct effects on adipose tissue that result in secondary beneficial effects on muscle and/or liver. The fact that PPARγ agonists lower circulating FFAs, presumably via increased FATP1 and FAT/CD36 levels in adipose tissue and possibly a decrease in lipolysis, is consistent with this hypothesis (17).

Other than changes in circulating FFAs, it is likely that additional effects on adipose tissue could contribute to metabolic efficacy. Among a number of known proteins that are expressed in adipocytes, we recently obtained preliminary data suggesting that adipocyte complement-related protein of 30 kDa (Acrp30) might be a gene that could be modulated by PPARγ (18). Acrp30, also known as adiponectin or AdipoQ, is a fat-specific expressed gene that encodes a secreted protein that circulates in plasma (19). Importantly, Acrp30 has also been recently implicated as a factor which

Abbreviations: Acrp, Adipocyte complement-related protein of 30 kDa; aP2, adipocyte fatty acid binding protein; BMI, body mass index; FATP1, fatty acid transport protein; HFD, high fat diet; TZDs, thiazolidinediones.

can mediate FFA lowering, enhanced *in vivo* insulin sensitivity and glucose lowering in rodents (18, 20, 21).

Here, we have systematically analyzed the PPARγ-mediated induction of Acrp30. Treatment of 3T3-L1 preadipocytes with PPARγ agonists induces Acrp30 mRNA expression. White adipose tissue Acrp30 mRNA expression was induced after chronic treatment of diabetic mice with PPARγ agonists. Using a polyclonal Acrp30 antibody, we demonstrated that plasma Acrp30 levels from two diabetic mouse models were increased in response to PPARγ agonist treatment. As there are currently no existing specific circulating biomarkers of PPARγ agonist effects in humans, we sought to determine if plasma Acrp30 protein levels would be affected by treatment of human subjects with a PPARγ agonist. After treatment of normal volunteers with rosiglitazone, a significant increase in Acrp30 levels was observed. Finally, we demonstrated for the first time that circulating Acrp30 levels were substantially reduced in patients with dominant-negative PPARγ mutations. Our results suggest that induction of Acrp30 may represent a key mechanism that contributes to the beneficial metabolic effects of PPARγ agonists and that measurement of Acrp30 levels may prove to be a valuable biomarker that can be used to gauge the extent of *in vivo* PPARγ activation in humans.

Materials and Methods

Culture and treatment of 3T3-L1 preadipocytes

3T3-L1 cells (ATCC, Manassas, VA; passages 3–9) were grown to confluence in medium A (DMEM with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 C in 5% CO_2 as previously described (22). Differentiation was induced by incubating the confluent fibroblasts with medium A supplemented with methylisobutylxanthine, dexamethasone, and insulin for 2 d, followed by another 2-d incubation with medium A supplemented with insulin. The cells were further incubated in medium A for an additional 3 d to complete the adipocyte conversion. RNA samples were prepared from fibroblasts, preadipocytes treated ± indicated ligands in the differentiation medium for 6 or 48 h, or fully differentiated adipocytes in medium A ± ligands for 6 or 48 h.

In vivo animal studies

Male *db/db* mice (10- to 11-wk-old C57Bl/KFJ, The Jackson Laboratory, Bar Harbor, ME) were housed 7/cage and allowed *ad libitum* access to ground Purina rodent chow and water. The animals, and their food, were weighed every 3 d and were dosed daily by gavage with vehicle (0.25% carboxymethylcellulose) ± PPAR agonists at the indicated doses. Plasma glucose and triglyceride concentrations were determined from blood obtained by tail bleeds at 3- to 4-d intervals during the studies. Glucose and triglyceride determinations were performed using assay kits with glucose oxidase for glucose (Sigma, St. Louis, MO) and glycerol kinase for triglycerides (Roche Molecular Biochemicals, Indianapolis, IN), respectively. Lean animals were age-matched heterozygous mice maintained in the same manner.

A nongenetic mouse model of diabetes (23) was generated as follows. Four-week-old male ICR mice (Taconic, Germantown, NY) were fed with high fat diet (HFD, 36% w/w, 58.4% kcal%, Research Diets, Inc., New Brunswick, NJ; D00031501) for 3 wk followed by a single ip administration of STZ at 100 mg $\times$ kg^{-1} BW. Animals were fed HFD for an additional 4 wk. Mice fed regular chow and injected with saline were used as controls. The HFD/STZ mice were treated twice daily with oral gavage of vehicle (0.5% methylcellulose) or vehicle containing compounds. Blood glucose was monitored with a glucometer (OneTouch Basic, Lifescan, Newtown, PA). Other parameters were measured as described above.

Human clinical study protocol

This was a single center, open-labeled, randomized, placebo-controlled, balanced, incomplete block, 4-treatment, 3-period crossover pilot study with the four treatments consisting of placebo, fenofibrate (201 mg once a day), fenofibrate (201 mg once a day) plus rosiglitazone (4 mg bid), and rosiglitazone (4 mg twice daily). The duration of treatment was 14 d, and there was at least a 14-d washout between periods. Each subject participated in three treatment periods in balanced fashion such that nine subjects received each treatment. Plasma for Acrp30 concentration determination was obtained predose on d 1 (baseline) and 2 h after the last dose on d 14 for each period. All 12 subjects were healthy males who varied in age from 18–42 yr (mean age 24 yr) and in weight from 61–110 kg (mean weight 89 kg). These subjects refrained from all other medication use from 14 d before completion of the trial. They had no evidence or family history of diabetes mellitus, baseline fasting plasma glucose was $<$ 110 mg/dl and $<$ 140 mg/dl 2 h after a 75-g oral glucose load, and baseline the baseline fasting plasma lipid profile (including triglycerides and total cholesterol) was within the reference range for the laboratory. All subjects gave written informed consent and the clinical protocol was reviewed by and approved by Schulman Associates Institutional Review Board (Cincinnati, OH). This clinical study was conducted according to the Declaration of Helsinki principles.

The log percent change [*i.e.* log(post/pre)] in Acrp30 levels was analyzed by using an analysis of covariance model, appropriate for balanced, incomplete block, 4-treatment, 3-period crossover. The final analysis of covariance model contained factors for subject, period, treatment, and baseline Acrp30 level (at the start of each treatment period). To assess the magnitude of the effect between the active treatment groups and placebo, 95% confidence intervals on the least square mean difference for the log percent change from baseline were also computed (24). All data were back-transformed to the original percent change from baseline scale for presentation purposes. For the purpose of this analysis, no results were available for the combination group of fenofibrate and rosiglitazone treatment group.

Additional plasma samples were obtained from three patients with dominant-negative PPARγ mutations, three additional normal control subjects, and eight patients with severe insulin resistance without mutations in the coding region of PPARγ.

Measurement of mRNA expression

Total RNA was prepared from cells and tissue using the Ultraspec RNA isolation kit (Biotecx, Houston, TX) then DNase treated using DNA-free according to the manufacturer's protocol (Ambion, Inc. Austin, TX). RNA concentrations were quantitated with Ribogreen by following the supplier's directions (Molecular Probes, Inc., Eugene, OR). Expression levels of Acrp30, adipocyte fatty acid binding protein (aP2), and fatty acid transport protein (FATP1) mRNAs were quantified using quantitative fluorescent real time PCR. RNA was first reverse-transcribed using random hexamers in a protocol provided by the manufacturer (PE Applied Biosystems, Foster City, CA). Amplification of each target cDNA was then performed with TaqMan PCR Reagent Kits in the ABI Prism 7700 Sequence Detection System according to the protocols provided by the manufacturer (PE Applied Biosystems). The following primer/probe sets were used for the amplification step:

Gene	5' Primer (5'-3')	3' Primer (5'-3')	Probe (5'-3')
Acrp30	TGTTGGAATGACAG GAGCTGAA	CACACTGAAGCCTG AGCGATAC	CATAAGCGGCTTCTCCAG GCTCTCCT
aP2	CACCGCAGACGACA GGAAG	GCACCTGCACCAGG GC	TGAAGAGCATCATAACCCT AGATGGCGG
FATP1	TTCTGCGTATCTGCA AGA	CGCAGCTCTAGCCG AACAC	CGAGACCTCTTTGGCCTCT CTGTTCTGA

The levels of mRNA were normalized to the amount of 18S ribosomal RNA (primers and probes commercially available from PE Applied Biosystems) detected in each sample.

Measurement of Acrp30 protein levels in plasma

Acrp30 was measured by quantitative Western blotting. After SDS-PAGE, proteins were transferred to BA83 nitrocellulose (Schleicher & Schuell, Inc., Keene, NH). Nitrocellulose membranes were stained with

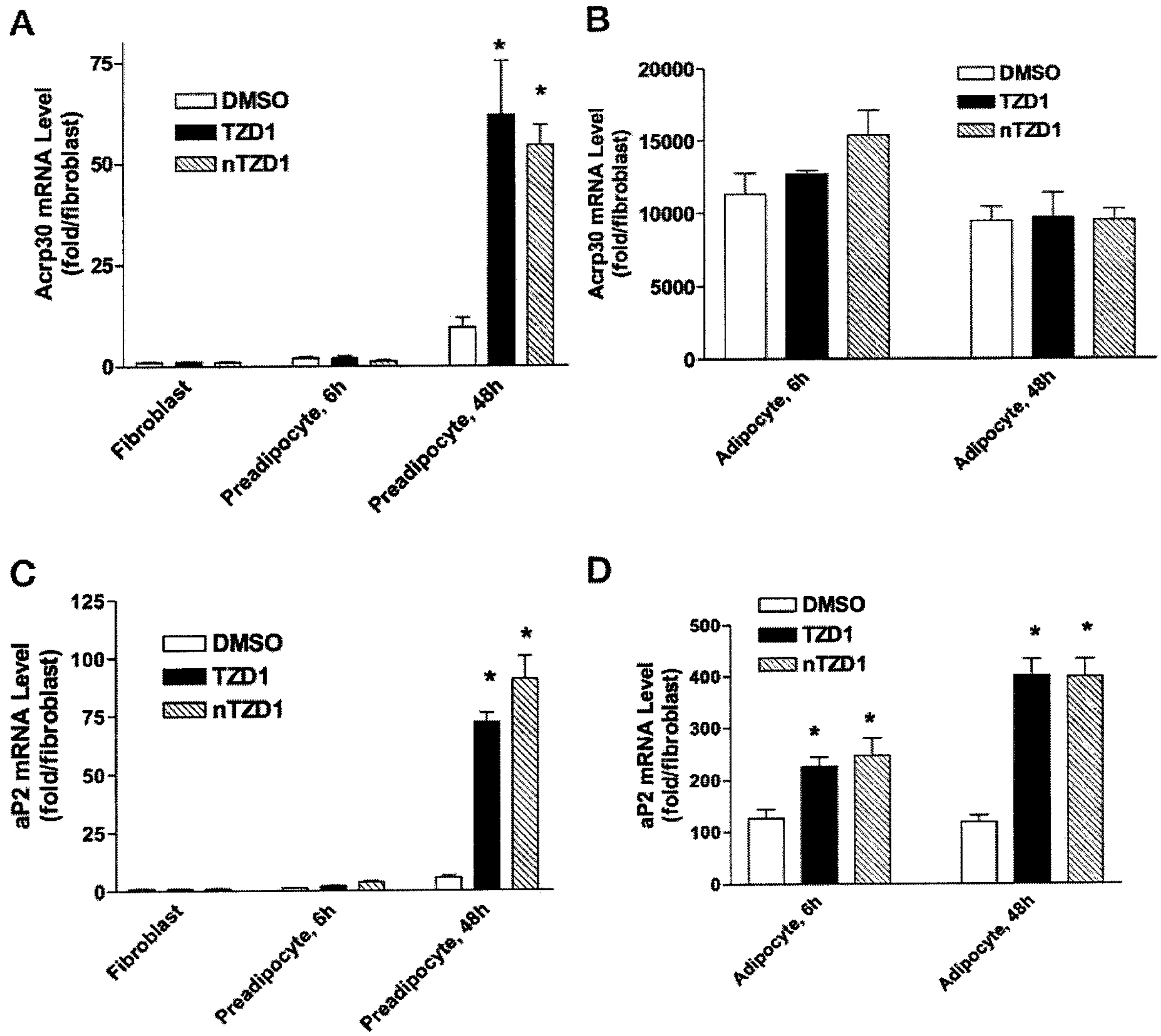


FIG. 1. Effect of PPARγ agonists on Acrp30 and aP2 gene expression in 3T3-L1 cells. 3T3-L1 preadipocytes (A, C) or fully differentiated adipocytes (B, D) were treated with either a thiazolidinedione (TZD1) or nonthiazolidinedione (nTZD1) PPARγ agonist. Acrp30 and aP2 mRNA levels were measured at indicated time points following treatment in comparison to cells exposed to DMSO control medium or to untreated fibroblasts. Mean (± SEM) of three determinations are shown. *, $P < 0.05$ *vs.* DMSO by *t* test. Similar results were obtained in additional experiments.

Ponceau S solution to ensure even and complete transfer of all samples and subsequently blocked in PBS or Tris-buffered saline with 0.1% Tween-20 and 5% nonfat dry milk. An affinity-purified rabbit antimouse Acrp30 antibody raised against a peptide comprising the hypervariable region (EDDVTTTEELAPALV) was used (19); this antibody recognizes a single band by Western blot analysis that can effectively be competed with excess immune peptide. This antibody was derivatized with ^{125}I and was used to decorate the blots. Each gel contained four standards of purified mouse Acrp30 at four different concentrations to ensure linearity and reproducibility of the signal. For the analysis of human serum samples, a rabbit antihuman Acrp30 antibody, directed against the hypervariable region of the human protein (DQETTTQGPGV), was employed. This antibody was visualized with an ^{125}I-derivatized secondary goat antirabbit antibody; a standardized human serum sample was also applied to each gel in four different concentrations. However, as no standard for recombinant human Acrp30 protein was included, human plasma levels are expressed as relative units/ml rather than as absolute values. Blots were analyzed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) and quantitated with ImageQuant software (Molecular Dynamics, Inc.). Intraassay variability was measured using the same serum sample with two replicates per blot, with standards, and quantitating the SE of the two measurements; the SE was 9%. Interassay variability based on the measurement of serum samples from the same experiment assayed at different times gave a SEM of 10–20%, emphasizing the high level of reproducibility of the assay. Lower limits of detection are 1–5 ng of Acrp30 protein. For protein extractions from adipose tissue and liver, 100 mg of tissue was mixed with 1 ml of ice-cold Tris-buffered saline containing protease inhibitors, sonicated for 10 sec, and centrifuged at 13,000 × *g* for 2 min. The fat cake was removed by suction, Triton X-100 was added to the infranatant to a final concentration of 1%, samples sonicated again for 10 sec, centrifuged at 13,000 × *g*, and the supernatant used for protein determinations by BCA (Pierce Chemical Co., Rockford, IL).

Results

PPARγ agonists mediate induction of Acrp30 gene expression in 3T3-L1 preadipocytes

To assess the effects of PPARγ activation on Acrp30 gene expression in 3T3-L1 cells, we employed two previously described compounds (10) with potent PPARγ agonist activity: a PPARγ-specific TZD (TZD1), 5-[4-[2-(5-methyl-2-phenyl-4-

oxazoly)-2-hydroxyethoxy]benzyl-2,4-thiazolidinedione, and a potent non-TZD, L-796449 (nTZD1). 3T3-L1 preadipocytes were incubated with 1 μM of TZD1 or nTZD1 *vs.* control medium for 6 h or 48 h. As depicted in Fig. 1A, Acrp30 mRNA was rapidly induced (~10×) during the first 48 h of differentiation and both PPARγ agonists produced a marked (~5-fold) further increase at the 48-h time point. Both compounds resulted in a similar increase in the expression of a well characterized PPARγ target gene, aP2 (Fig. 1C).

In fully differentiated 3T3-L1 adipocytes, the Acrp30 mRNA levels were induced by more than 1,000-fold compared with the level in undifferentiated fibroblasts. In contrast to the effects of PPARγ agonists on Acrp30 in preadipocytes, TZD1 and nTZD1 did not alter Acrp30 mRNA levels further in differentiated adipocytes (Fig. 1B). In comparison, aP2 mRNA levels were increased by approximately 100-fold in adipocytes relative to fibroblasts and treatment of adipocytes with TZD1 and nTZD1 resulted in further elevations of aP2 mRNA level (~2-fold at 6 h, ~4-fold at 48 h).

Effects of in vivo PPARγ agonist treatment on Acrp30 gene expression in white adipose tissue and plasma Acrp30 levels in db/db mice

Male *db/db* mice with overt hyperglycemia (mean plasma glucose levels > 600 mg/dl) were treated with rosiglitazone at a dose of 10 mg/kg·d via daily oral gavage. Following 11 d of treatment, samples of white adipose tissue (epididymal) and plasma were obtained (24 h after the last dose of rosiglitazone). Table 1 shows d-11 values for glucose, triglycerides, and body weight in rosiglitazone-treated mice *vs.* *db/db* mice treated only with vehicle and lean control mice. Rosiglitazone treatment was associated with 50% reduction in glycemia and reduced triglyceride levels below those observed in lean db/+ mice. Body weights increased with rosiglitazone treatment by approximately 10%.

Measurement of adipose tissue Acrp30 mRNA by quantitative RT-PCR revealed that Acrp30 mRNA levels in vehicle-treated *db/db* control mice were approximately 50% lower than in lean control mice (Fig. 2A). Rosiglitazone treatment of *db/db* mice was associated with a significant (*vs.* vehicle) increase in mean Acrp30 mRNA levels. Thus, TZD treatment resulted in levels of adipose Acrp30 mRNA that approached levels in lean mice. As a control, we also examined the effect of rosiglitazone treatment on expression of FATP1, a fatty acid transport protein that is known to be a direct PPARγ target gene (17). Similar to Acrp30, FATP1 mRNA levels were significantly induced in adipose tissue following *in vivo* TZD treatment. (Fig. 2B). Similar results were obtained in three independent *db/db* mouse studies.

TABLE 1. *In vivo* effects of TZD treatment in *db/db* mice

	Lean *db/+*	*db/db* control	*db/db* + rosiglitazone
Body weight % increase over 11-d dosing	—	2.4	12
Triglycerides mg/dl	136 ± 15	248 ± 10[a]	90 ± 6[b]
Glucose mg/dl	153 ± 6	635 ± 36[a]	314 ± 19[b]

Male *db/db* mice (n = 7 per group) were treated with control vehicle or with 10 mg/kg·d with rosiglitazone via daily oral gavage for 11 d. Effects on body weight, triglycerides, and glucose are shown in comparison with values from lean control *db/+* mice.

[a] $P < 0.0001$ *vs.* lean; [b] $P < 0.0001$ *vs.* *db/db* vehicle control.

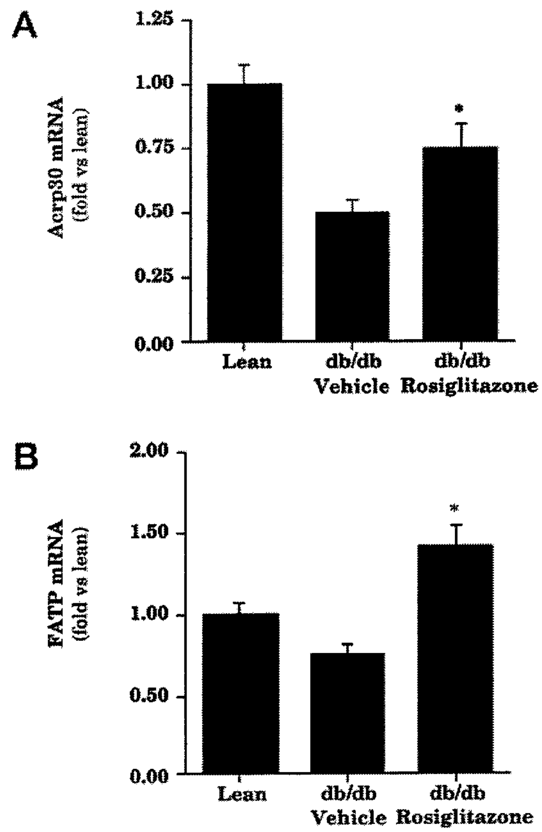
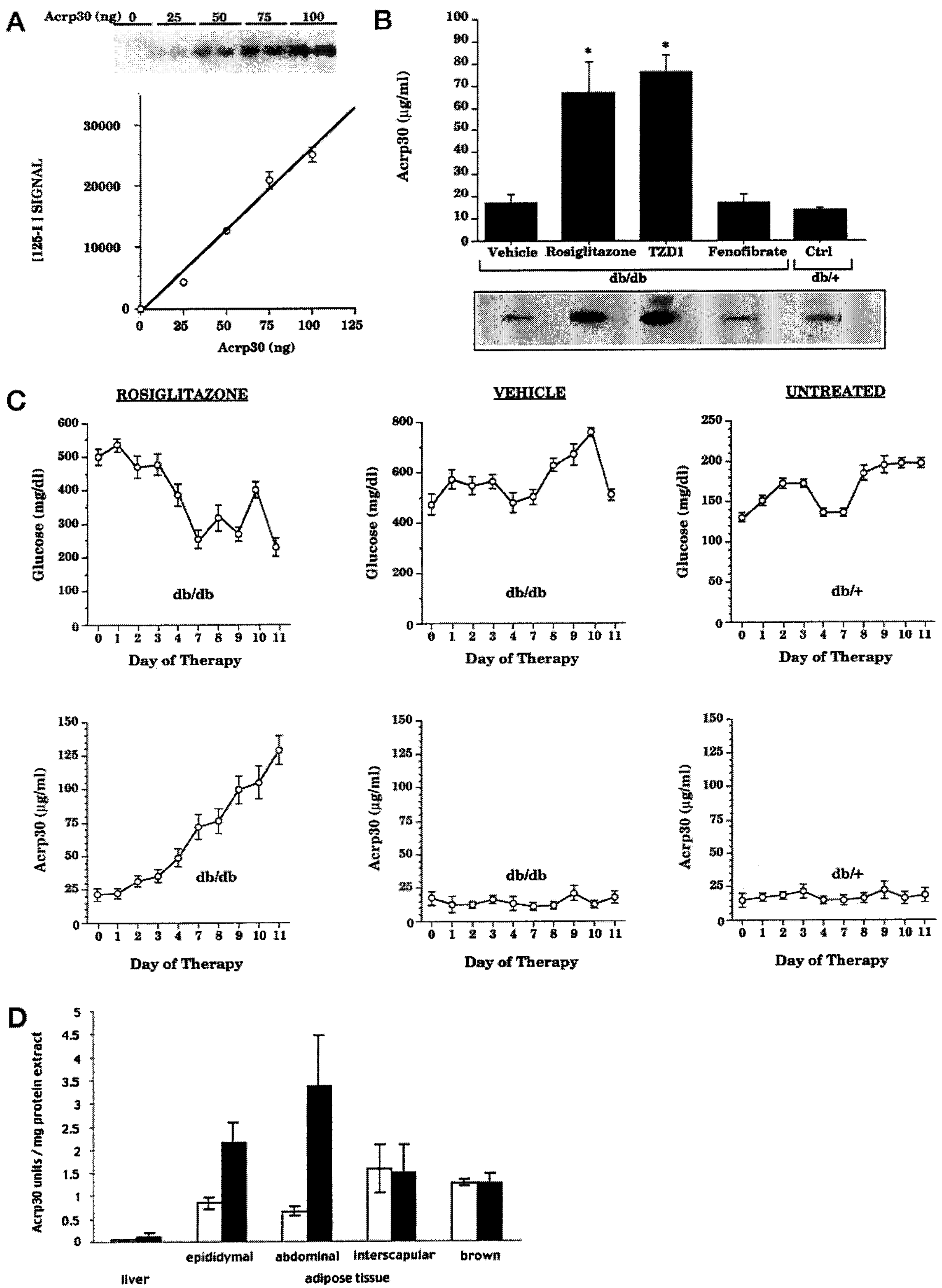


FIG. 2. Effect of *in vivo* treatment with PPARγ agonist on white adipose tissue Acrp30 mRNA expression in *db/db* mice. Rosiglitazone was administered to *db/db* mice for 11 d. Total RNA was isolated from epididymal white adipose tissue of the lean controls, vehicle-treated *db/db* controls, and *db/db* treated with rosiglitazone (3 mg/kg·d). The levels of Acrp30 (A) and FATP1 (B) mRNA expression were quantified by quantitative real-time PCR and are expressed relative to the amount of mRNA found in the lean controls. The data are shown as the means ± SEM of seven individual samples from each treatment group. *, $P < 0.05$ comparing data to vehicle-treated *db/db* by *t* test. Similar results were obtained in two additional experiments.

To assess the effects of *in vivo* rosiglitazone treatment on circulating plasma levels of Acrp30, we developed a quantitative immunoblot method using a polyclonal Acrp30 antibody. To illustrate linearity of the Western blot method, increasing amounts of HEK293-T produced and purified Acrp30 (18) were analyzed by quantitative immunoblotting (Fig. 3A). Each SDS-PAGE gel contained a similar set of four standards to allow quantitative comparison of samples on separate gels. The linearity of the signal was also tested using incremental increases in serum sample amount (not shown). Plasma samples from two separate *db/db* mouse studies were analyzed. As depicted in Fig. 3B, mean Acrp30 levels in *db/db* mice increased by approximately 3- to 4-fold after TZD1 or rosiglitazone treatment *vs.* vehicle. Treatment with a PPARα agonist, fenofibrate (150 mg/kg·d), did not lower glucose or induce an increase in Acrp30 levels (Fig. 3B) despite a significant (27%) decline in mean triglyceride levels (not shown).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[125-I] SIGNAL
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Vehicle Rosiglitazone TZD1 Fenofibrate Ctrl
db/db
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ROSIGLITAZONE
VEHICLE
UNTREATED
Glucose (mg/dl)
db/db
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Day of Therapy
0 1 2 3 4 7 8 9 10 11
Acrp30 (μg/ml)
db/db
db/db
db/+
Day of Therapy
D
Acrp30 units / mg protein extract
5 4.5 4 3.5 3 2.5 2 1.5 1 0.5 0
liver
epididymal
abdominal
interscapular
brown
adipose tissue

To determine whether the elevation of circulating Acrp30 levels precedes, coincides with, or follows the decrease in serum glucose levels, a more detailed time-course study was performed. Rosiglitazone or vehicle treatment was administered daily to *db/db* mice for a period of 11 d (Fig. 3C). During that period, serum glucose and Acrp30 levels were measured at several intervals. Serial blood samples were also obtained from a parallel group of lean db/+ control mice. In vehicle-treated *db/db* mice and in untreated lean mice, serum Acrp30 and glucose levels remained unchanged. On fourth and subsequent days of dosing with rosiglitazone, plasma glucose levels were significantly decreased ($P < 0.05$). Plasma Acrp30 levels were significantly higher on the third and subsequent days of dosing with rosiglitazone. Glucose levels were significantly reduced by d 4 of therapy with further improvement thereafter. Acrp30 levels, in turn, were increased (40%) between d 1 and d 2, with further increases observed between d 3 and d 7 (>3-fold increase). Acrp30 levels continued to increase linearly throughout the 11-d regimen resulting in 6-fold elevation by the end of the study compared with initial levels. These results indicate that increased serum Acrp30 levels may precede (or at least coincide with) correction of hyperglycemia in this insulin-resistant murine model.

To confirm that rosiglitazone treatment leads to an upregulation of Acrp30 protein within various fat pads, protein was extracted from epididymal, abdominal, interscapular white, and interscapular brown adipose tissue as well as liver from both vehicle- and rosiglitazone-treated animals at the end of the experiment. Protein extracts were analyzed by SDS-PAGE and quantitative immunoblotting for Acrp30 (Fig. 3E). Epididymal and abdominal pads responded to rosiglitazone treatment with a marked increase of Acrp30, suggesting that a significant fraction of the increase in serum Acrp30 levels is caused by increased production. Decreased clearance of serum Acrp30 therefore cannot exclusively account for the increase in circulating Acrp30. As expected, the liver (not perfused) did not have significant amounts of Acrp30, even after rosiglitazone treatment. Surprisingly, production of Acrp30 in interscapular white and brown adipose tissue was not affected by rosiglitazone treatment, suggesting a fat depot specific response to rosiglitazone action.

PPARγ agonist-mediated induction of Acrp30 plasma levels in a nongenetic rodent model of type 2 diabetes

Additional *in vivo* experiments were conducted using a nongenetic model of type 2 diabetes. Combined low-dose streptozotocin treatment and high-fat feeding (HFD/STZ) of ICR mice resulted in moderate hyperglycemia *vs.* control ICR mice on a normal chow diet (see Table 2). Twice daily oral gavage treatment of HFD/STZ mice for 11 d with 10 mg/kg doses of rosiglitazone or an additional potent PPARγ selective non-TZD compound, L-805645 (nTZD2) (25) resulted in substantial correction of hyperglycemia (Table 2). In addition, a separate group of HFD/STZ mice received metformin (at a dose of 200 mg/kg given twice daily) as an alternative antidiabetic therapy that is known to be independent of PPARγ activity. Treatment with both PPARγ agonists and metformin resulted in significant reduction in hyperglycemia. Plasma samples were obtained on d 11 and subsequently analyzed for determination of Acrp30 protein levels. As depicted in Fig. 4, mean Acrp30 levels were substantially increased by treatment with either the TZD or non TZD PPARγ agonist. In contrast, metformin had no effect on mean Acrp30 plasma levels.

TABLE 2. Glucose-lowering effects of PPARγ agonists and metformin in HFD/STZ mouse model

	Glucose (mg/dl)
Lean (chow/saline)	149 ± 6
HFD/STZ	
Vehicle	469 ± 17[a]
Rosiglitazone (10 mpk)	271 ± 51[b]
nTZD2 (10 mpk)	294 ± 44[b]
Metformin (200 mpk)	291 ± 40[b]

Male ICR mice were induced to develop type 2 diabetes by combination of HFD/STZ treatment. Mice were treated twice daily by oral gavage with vehicle or test compounds for 11 d. Blood glucose levels were measured at 4 h post last dose. Data shown represents mean ± SEM. mpk, mg/kg·d.

[a] $P < 0.01$, comparing lean *vs.* HFD/STZ group (n = 6–8 in each group).

[b] $P < 0.05$, comparing treatment group with the vehicle control group.

Treatment of human subjects with rosiglitazone results in the induction of mean plasma Acrp30 levels

Healthy male subjects were treated with rosiglitazone at a dose of 4 mg orally twice daily for 14 d. Similarly, these subjects were treated orally with placebo once daily for 14 d, fenofibrate 201 mg orally once daily, and the combination of rosiglitazone and fenofibrate (not assessed in this study). As shown in Fig. 5, rosiglitazone treatment for 14 d produced an approximately 130% least square mean increase from baseline in circulating Acrp30 levels *vs.* placebo (statistically significant at $P = 0.010$). In contrast, fenofibrate treatment had no significant effect on mean Acrp30 levels.

Patients with dominant-negative PPARγ mutations have suppressed Acrp30 levels

We previously reported two dominant-negative PPARγ mutations (Val[290]-Met and Pro[467]-Leu) that occurred in association with severe insulin resistance and type 2 diabetes

FIG. 3. A, Western blot analysis of increasing amounts of HEK293-T-produced Acrp30. B, Serum Acrp30 levels in *db/db* mice after treatment with either of two PPARγ agonists. Male *db/db* mice (n = 7, each group) were treated with rosiglitazone (10 mg/kg·d), TZD1 (10 mg/kg/d), fenofibrate (150 mg/kg·d), or control vehicle for 11 d followed by measurement of serum Acrp30 levels. Mean ± SEM values are depicted with representative samples shown below. Serum Acrp30 levels obtained from control (Ctrl) lean (*db/+*) mice are shown for comparison. *, $P < 0.05$ *vs.* vehicle. C, Temporal effects of rosiglitazone *vs.* vehicle treatment in *db/db* mice on serum Acrp30 (μg/ml) and plasma glucose (mg/dl) levels. *Left panels*, Rosiglitazone-treated *db/db* mice; *center panels*, vehicle-treated *db/db* mice; *right panels*, results from untreated control *db/+* mice are shown for comparison. Each *point* represents the mean (± SEM) of seven individual animals. D, Acrp30 protein levels in vehicle-treated (*white bars*) and rosiglitazone-treated (*black bars*, 11-d treatment) tissues. Mean ± SEM values are depicted.

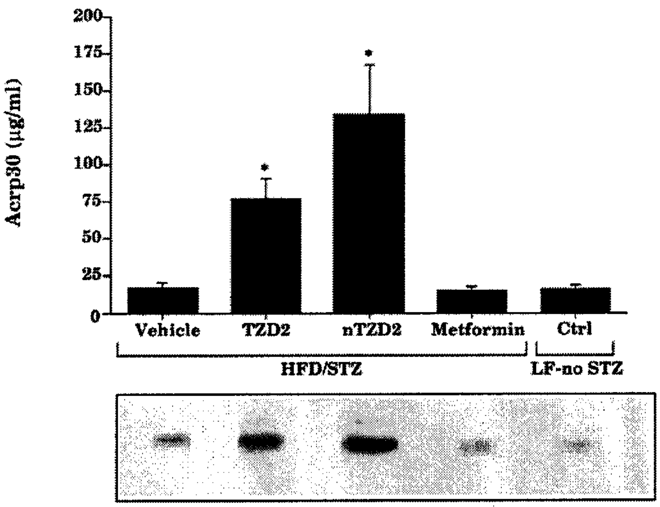

FIG. 4. Effects of rosiglitazone or nTZD2 PPARγ agonists on plasma Acrp30 levels in a nongenetic murine model of type 2 diabetes. Acrp30 plasma levels were determined using samples obtained from high fat-fed plus streptozotocin-treated (HFD/STZ) mice (n = 6, each group) which were treated for 11 d with rosiglitazone or nTZD2 (10 mg/kg·d twice daily) or with metformin (200 mg/kg·d twice daily) *vs.* control vehicle. Serum Acrp30 levels obtained from control low-fat chow fed mice (LF-no STZ) are shown for comparison. Mean ± SEM values are depicted. *, $P < 0.05$ *vs.* vehicle.

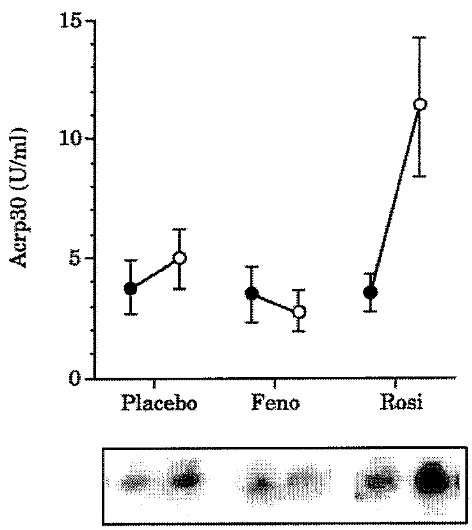

FIG. 5. Treatment of healthy human subjects with rosiglitazone produces a significant increase from baseline in mean Acrp30 plasma levels ($P = 0.010$ *vs.* placebo change from baseline). Effects of placebo, rosiglitazone (4 mg twice daily), or fenofibrate (201 mg once daily) on plasma Acrp30 levels in healthy male subjects (n = 8, placebo; n = 9, rosiglitazone and fenofibrate treatments). Acrp30 plasma levels were determined using plasma samples obtained predose on d 1 (*closed circles*) and 2 h after the last study drug dose on d 14 of treatment (*open circles*). Mean ± SEM values are depicted.

(12). Plasma Acrp30 levels were measured in two probands with the Pro^{467}-Leu allele and in one with the Val^{290}-Met allele (12). As shown in Table 3, Acrp30 levels in these patients were within a very low range (0.17–0.71 U/ml) compared with values measured at the same time in three unrelated normal control subjects and compared with placebo or pretreatment values measured in the experiments described for normal human subjects in Fig. 5. In addition, mean values from the three probands with dominant-negative mutations were significantly lower (5-fold) than the mean of values measured with samples from 8 unrelated patients with severe insulin resistance in whom no mutations in the PPARγ coding region were detected (a subset of the 85 patients studied in Ref. 12). Furthermore, the plasma Acrp30 level remained below "normal" following 12 wk of treatment of the Val^{290}-Met patient with rosiglitazone (Table 3). This is consistent with the fact that this mutation renders the receptor nearly unresponsive to activation by rosiglitazone *in vitro* (12). Although body mass index (BMI) values tended to be lower in the three probands than in the other study subjects, there was no significant correlation between Acrp30 and BMI in the larger cohort of insulin resistant patients (subjects 7–14 in Table 3). Not every decrease of Acrp30 levels in diabetic patients is caused by a mutation in PPARγ because a number of epidemiological studies have reported a decrease in parallel with reduced insulin sensitivity during the progression to type 2 diabetes (26, 27). Some of patients 7–14 will most likely display decreased Acrp30 levels for reasons unrelated to mutations in PPARγ. In summary, upon ranking circulating Acrp30 levels of all these patients, it is striking that all three patients with known mutations interfering with PPARγ activity display the lowest serum Acrp30 levels.

Discussion

The cDNA encoding a novel protein, adipocyte complement-related protein of 30 kDa (Acrp30), was initially identified by subtractive cloning or mRNA differential display from cultured adipocytes *vs.* preadipocytes (19, 28). Acrp30 is a protein of 247 amino acids that bears homology to complement factor C1q; it contains both an amino-terminal collagenous domain and a C-terminal globular domain. Interestingly, the x-ray crystal structure of the globular domain shows it to be similar to that of TNFα (29). The distribution of Acrp30 mRNA in mouse, rat, and human is confined almost exclusively to adipose tissue and its expression is strongly induced during differentiation of cultured preadipocytes (3T3-L1 or 3T3-F442A) mediated by a cocktail containing dexamethasone, isobutylmethylxanthine, and insulin (19, 28). Hu *et al.* (28) also showed that Acrp30 mRNA expression levels were reduced in white adipose tissue derived from obese mice (*ob/ob*) or humans *vs.* fat tissue from lean controls. Importantly, Acrp30 is a secreted protein that circulates in plasma at high concentrations (5–10 μg/ml) (19). These observations suggested that Acrp30 represents a circulating adipose-derived factor, like leptin, which could influence energy balance and/or metabolic perturbations that are associated with obesity. Several recent reports have functionally linked Acrp30 to improved systemic insulin sensitivity (18, 20, 21). Lodish and colleagues (20) reported that acute administration of recombinant Acrp30 lowered postprandial levels of glucose, triglycerides, and FFAs in mice fed with high-fat and sucrose. Greater efficacy was seen with a truncated 27-kDa version of the protein containing the globular head domain than with the full-length protein; however,

TABLE 3. Acrp30 levels in patients with dominant-negative PPARγ mutations and other forms of severe insulin resistance

Subject no.	Age/sex	BMI (kg/m^2)	Phenotype	PPARγ genotype	Acrp30 level (U/ml)
1	56/F	24.4	IR	P467L	0.71
2	32/M	24.9	IR	P467L	0.25
3	21/F	28.6	IR	V290M	0.17
					Mean 0.38 ± 0.05[a,b]
3(A)	21/F		IR	V290M	0.24
4	37/F	42.6	Normal	Normal	1.75
5	50/F	34.9	Normal	Normal	1.49
6	71/F	32.9	Normal	Normal	3.46
					Mean 2.23 ± 0.66
7	24/M	36.4	IR	Normal	4.75
8	22/F	49.6	IR	Normal	1.72
9	20/F	28	IR	Normal	1.90
10	23/F	37.2	IR	Normal	0.90
11	11/M	29.4	IR	Normal	0.86
12	5/F	34.1	IR	Normal	0.76
13	64/F	45.6	IR	Normal	2.38
14	17/M	32.1	IR	Normal	2.15
					Mean 1.93 ± 0.60

Acrp30 levels were determined using plasma samples obtained from three subjects (1–3) with severe insulin resistance (IR) and PPARγ dominant-negative mutations. A second sample from subject 3(A) was obtained after chronic therapy (12 wk) with rosiglitazone. Samples from three additional control subjects (4–6) and from eight patients with severe insulin resistance known to have no mutations within the PPARγ coding region (7–14) were also used. BMI values for each subject are shown. Individual and mean (± SEM) Acrp30 values are shown.

[a], $P = 0.02$ *vs.* mean of samples 4–6; [b], $P = 0.04$ *vs.* mean of samples 7–14 (one-tailed *t* test).

postprandial glucose levels were similarly affected by both forms of Acrp30 in this experimental paradigm. In addition, an effect of the 27-kDa protein to induce fatty acid oxidation in cultured skeletal muscle cells was described. In line with these observations, Kadowaki and colleagues (21) showed that Acrp30 leads to the increased expression of molecules involved in fatty acid oxidation and energy dissipation in muscle and were able to reverse insulin resistance in lipoatrophic mice only upon combined administration of Acrp30 and leptin. We have recently observed that single injections of full-length Acrp30 produced lowered glucose levels in both normal, *ob/ob* mice and NOD mice, independently of an increase in insulin levels (18). The fact that recombinant Acrp30 could also enhance the effect of insulin to suppress glucose output from cultured hepatocytes suggests that a primary effect of this protein may be to augment hepatic insulin action.

A study by Tataranni and colleagues (26) in Caucasians and Pima Indians (a population with a high propensity for obesity and type 2 diabetes) further corroborates the relationship with metabolic parameters. After multivariate analysis, the authors concluded that decreased plasma Acrp30 levels ("hypoadiponectinemia") are more closely related to the degree of insulin resistance and hyperinsulinemia than to the degree of adiposity and glucose intolerance. Furthermore, Comuzzie *et al.* (30) have found that the Acrp30 gene locus (3q27) demonstrates significant LOD scores influencing phenotypic aspects of metabolic syndrome X.

In the present studies, we sought to further explore whether Acrp30 mRNA or protein levels could be affected by PPARγ activation, and how the Acrp30 induction is kinetically related to the TZD-mediated reduction of serum glucose levels. We initially determined that PPARγ agonists strongly induced Acrp30 mRNA expression in cultured 3T3-L1 preadipocytes. As similar results were obtained using both a TZD and a non-TZD PPARγ ligand and because stimulation of Acrp30 was observed with concentrations of these compounds that were within a 10-fold range of their respective PPARγ binding affinities, it is apparent that PPARγ activation *per se* was sufficient to produce this effect (along with other aspects of adipocyte differentiation). The question whether Acrp30 is a gene that is directly transactivated by PPARγ via a PPARγ-responsive elements in its promoter remains an important one. We previously cloned and sequenced the murine Acrp30 promoter region (31). In several kilobase pairs of this region and in the first intron, there are no consensus PPARγ-responsive elements sites. Potential sites for CCAAT/enhancer binding protein β (C/EBPβ) suggest a potential mechanism for induction of Acrp30 during adipocyte differentiation and/or as a secondary mechanism by which PPARγ might induce Acrp30 gene expression. In agreement with the latter hypothesis, TZDs failed to further induce Acrp30 in isolated, mature 3T3-L1 adipocytes. However, the studies presented here are not focused on this question and do not allow us to conclusively resolve this issue. Rather, we focused on the hypothesis that circulating Acrp30 levels might be modulated in relation to *in vivo* PPARγ activation.

The mechanism(s) by which PPARγ activation leads to an increase in circulating Acrp30 levels may therefore include the following: 1) as discussed above, Acrp30 may be a directly affected PPARγ target gene; 2) activation of PPARγ *in vivo* is likely to promote an increase in adipogenesis (32) that may result in a greater net capacity for Acrp30 production; 3) posttranslational mechanisms may include the fact that insulin can promote Acrp30 exocytosis in a PI3K-dependent fashion (33) because we and others have shown that PPARγ agonists can selectively augment insulin-mediated PI3K ac-

tivity (34). However, both the magnitude of the induction as well as the observation that elevated levels are sustained during the treatment suggests that a purely posttranslational mechanism is unlikely. The rapid induction of Acrp30 levels after initiation of TZD treatment also suggests that an increase in the number of new adipocytes could not fully account for this phenomenon, at least not at the early timepoints of treatment. Furthermore, increased adiposity generally does not lead to an increase in Acrp30 levels in serum (18, 35); 4) lastly, the net increase in serum Acrp30 levels could be caused by decreased clearance. While we cannot eliminate a net contribution of decreased clearance, the increased presence of Acrp30 in fat pads suggests a net increase in Acrp30 protein production.

Administration of fenofibrate (to *db/db* mice), a PPARα-selective agonist, did not produce an increase in Acrp30 levels despite a significant effect to suppress elevated triglycerides. More importantly, treatment of HFD/STZ mice with a non-PPAR-associated antihyperglycemic agent, metformin, produced significant efficacy without affecting mean Acrp30 levels. These observations have very important implications for our understanding of the regulation of Acrp30 expression. They demonstrate that the effect of insulin sensitizers on *in vivo* Acrp30 levels is indeed (directly or indirectly) PPARγ mediated, and that changes in Acrp30 are not simply a consequence of an improved metabolic phenotype.

In agreement with the observations reported in this paper, Matsuzawa and colleagues (36) recently reported that the administration of TZDs significantly increased the plasma adiponectin (also known as Acrp30) concentrations in insulin-resistant humans and rodents. They demonstrated that adiponectin mRNA expression was normalized or increased by TZDs in the adipose tissues of obese mice. However, in contrast to our observations, they showed that cultured 3T3-L1 adipocytes enhanced the mRNA expression and secretion of adiponectin in a dose- and time-dependent manner in response to TZDs. We cannot fully explain these differences, but a likely explanation lies in the use of different clonal isolates of 3T3-L1 cells that may differ with respect to their ability to induce the factor(s) that critically mediate the TZD-induced transcriptional changes with respect to Acrp30 induction.

At present, there are no known human biomarkers that are specific for *in vivo* activation of PPARγ as well as useful in healthy volunteers or patients with type 2 diabetes. In patients with type 2 diabetes, measures of glucose metabolism are useful surrogates, but these measures are not specific to activation of PPARγ. Such biomarkers of activation of PPARγ, if affected in a short time-frame, would be invaluable in helping to determine whether individual patients are responding to treatment with PPARγ agonists and in aiding in the quantitation of *in vivo* PPARγ tone as a potential contributor to specific metabolic disease states. A key finding of the present study is the clear effect of *in vivo* treatment with a therapeutic dose of rosiglitazone to significantly increase mean Acrp30 levels in a placebo-controlled trial with normal human subjects. It is important to note that other metabolic parameters such as insulin, glucose, and FFAs were not affected in a statistically significant fashion by rosiglitazone in these otherwise healthy, normal volunteers (data not shown). In addition, such metabolic effects of rosiglitazone and related TZD PPARγ agonists that have been previously reported to occur in patients with type 2 diabetes typically occur more gradually, over the course of 6–12 wk (7, 37). As part of this exploratory clinical trial, subjects also received treatment with a therapeutic dose of fenofibrate. As we observed in mice, fenofibrate did not affect circulating Acrp30 levels in humans. Given these observations, it is apparent that increased Acrp30 is a robust, relatively early, specific response to activation of PPARγ in humans. A detailed human time course experiment is necessary to demonstrate that Acrp30 increases precede overt changes in the *in vivo* metabolic milieu, although the time course results in *db/db* mice are suggestive (see Fig. 3). Acrp30 represents a novel and potentially important biomarker for PPARγ activation. Further studies are required to determine whether PPARγ-mediated effects on Acrp30 occur in patients with type 2 diabetes, assess whether improvements in insulin sensitivity correlate with Acrp30 induction, and define a PPARγ agonist dose-response relationship for Acrp30.

The Pro^{467}-Leu or Val^{290}-Met PPARγ mutations are known to encode receptors with severely reduced function and dominant-negative properties *in vitro* (12). Thus, our observation that circulating Acrp30 levels were suppressed in severely insulin-resistant patients with either of these mutant alleles provides strong evidence that normal physiologic degrees of PPARγ activation regulate Acrp30 levels in humans. The role of PPARγ in mediating the effect of rosiglitazone to induce Acrp30 *in vivo* in humans was also bolstered by finding that chronic rosiglitazone treatment failed to substantially induce Acrp30 in a patient with the Val^{290}-Met mutation.

The recent reports cited above that demonstrate that Acrp30 can modulate systemic insulin sensitivity support the hypothesis that PPARγ agonists mediate an increase in circulating Acrp30 protein levels that, in turn, produces effects on liver and muscle that may act in concert to cause several of the well described net *in vivo* effects of TZDs: hepatic insulin sensitization, glucose lowering, FFA lowering, and (at least in rodents) triglyceride lowering. Given that suppressed Acrp30 levels were observed in patients with dominant-negative PPARγ mutations, it is also tempting to speculate Acrp30 deficiency has an important role in the pathogenesis of severe insulin resistance and type 2 diabetes that is present in these same patients.

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